

Effect of fibrinolysis on bleeding phenotype in moderate and severe von Willebrand disease

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ORIGINAL ARTICLE *Laboratory science*

Effect of fibrinolysis on bleeding phenotype in moderate and severe von Willebrand disease

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Summary. Patients with von Willebrand disease (VWD), the most common inherited bleeding disorder, display large variation in bleeding tendency, which is not completely related to VWF levels. The cause of variability in clinical expression is largely unknown. The effect of plasma fibrinolytic capacity on bleeding tendency in VWD patients has not been investigated. We hypothesized that enhanced fibrinolysis may result in a more severe bleeding phenotype. Therefore, we measured the fibrinolytic potential in patients with moderate or severe VWD to investigate the contribution of fibrinolysis to the bleeding tendency. Fibrinolytic potential was measured as plasma clot lysis time (CLT) with and without addition of potato carboxypeptidase inhibitor (PCI) in 638 patients with moderate or severe VWD who participated in a nationwide multicentre cross-sectional study.

Bleeding severity was measured using the Bleeding Score (BS). The CLTs were significantly longer, indicative of hypofibrinolysis, in men compared to women with VWD [106.2 (IQR 95.7–118.1) vs. 101.9 (IQR 92.8–114.0) min]. The CLTs prolonged with increasing age. No association was found between VWF or FVIII levels and CLT, or between VWF or FVIII levels and CLT^{PCI}. No association was observed for BS in a model with 10log-transformed CLT, adjusted for age, gender, VWF:Act and FVIII [$b = 6.5$ (95%CI -0.3 to 13.4)]. Our study showed that the plasma fibrinolytic potential does not influence bleeding tendency in VWD patients and therefore does not explain the variability in bleeding phenotype in VWD.

Keywords: bleeding, fibrinolysis, von Willebrand disease

Introduction

von Willebrand disease (VWD) is the most common inherited bleeding disorder, caused by defects in or reduced levels of von Willebrand factor (VWF) [1]. The VWF plays a major role in primary haemostasis by

facilitating adhesion of platelets to the endothelium, thereby initiating aggregation of platelets to form a platelet plug. In addition, VWF is the carrier protein of factor VIII (FVIII) [2].

In patients with VWD, VWF and FVIII levels largely determine the bleeding tendency, however, the variation in bleeding tendency between individuals with VWD is not completely related to VWF levels [3]. Some patients bleed excessively, whereas others with similar VWF levels in plasma have only mild bleeding problems. Also within families with similar mutations, large differences in bleeding phenotype are observed [3]. The cause of this variability in clinical expression of VWD is largely

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unknown. It has recently been shown that variability in thrombin generation may lead to differences in bleeding phenotype in VWD [4].

Another factor that may determine the variability in clinical expression of VWD is the rate of fibrinolysis. The effect of fibrinolysis on the bleeding tendency in VWD patients has not yet been investigated. The fibrinolytic system converts plasminogen into plasmin, which degrades the insoluble fibrin clot into soluble fibrin degradation products. Thrombin activatable fibrinolysis inhibitor (TAFI) connects the coagulation cascade with the fibrinolytic system. Upon high concentrations of thrombin, TAFI is activated by thrombin and effectively inhibits fibrinolysis by removing carboxyterminal lysine residues from partially degraded fibrin, thereby diminishing the cofactor function of fibrin in plasminogen activation [5].

Recent studies have indicated that the fibrinolytic potential, measured by a clot lysis assay, show considerable inter-individual variation [6]. This variability may also influence the bleeding phenotype of VWD individuals. In addition, in severe VWD patients the fibrinolytic potential may be altered by strongly reduced FVIII levels, leading to impaired thrombin generation via the intrinsic feedback-loop and less TAFI activation. Therefore, reduced FVIII levels in VWD not only have an effect on coagulation but may also enhance fibrinolysis. Previous studies have already indicated that TAFI levels and activation correlate with the fibrinolytic potential in healthy individuals [7,8]. Patients with enhanced fibrinolysis predominantly present with mucocutaneous bleeding [9–11], such as menorrhagia, epistaxis and gum bleeding. These bleeding symptoms are also frequently observed in patients with VWD [12].

We hypothesized that differences in fibrinolytic capacity may influence the bleeding tendency among VWD patients, i.e. enhanced fibrinolysis may result in a more severe bleeding phenotype. Therefore, we measured the fibrinolytic potential in a large cohort of patients with moderate or severe VWD to investigate the role of fibrinolysis in bleeding phenotype of VWD patients.

Methods

Willebrand in the Netherlands study

We performed a nation-wide cross-sectional study among patients with moderate and severe VWD in the Netherlands, the 'Willebrand in the Netherlands' (WiN) study. Patients were recruited at all 13 Haemophilia Treatment Centres in the Netherlands.

We included patients diagnosed with type 1, type 2 and type 3 VWD who fulfilled both of the following inclusion criteria: (i) haemorrhagic symptoms or a family history of VWD; (ii) historically lowest levels

of VWF antigen (VWF:Ag) ≤ 30 U dL⁻¹ and/or VWF activity (VWF ristocetin cofactor activity (VWF:RCO) ≤ 30 U dL⁻¹ and/or factor (F)VIII:C ≤ 40 U dL⁻¹, determined at the local Haemophilia Treatment Centre.

For the current analysis we included all patients, both children and adults. Data were obtained between October 2007 and October 2009. All participants, or their parents, completed a questionnaire and a blood sample was obtained. The Medical Ethical Committee decided that in children blood could only be obtained if for clinical purposes a blood sample was needed. The Medical Ethical Committees at all participating centres approved this study, and written informed consent was obtained from all study participants and/or their parents.

Definitions

Determination of type of VWD was based on the current ISTH guidelines [13,14], using the laboratory parameters determined at the local Hemophilia Treatment Center. The severity of VWD was defined as proposed by Federici [15]. Severe VWD was defined as the presence of at least one of the following laboratory abnormalities: VWF:Ag < 10 U dL⁻¹, and/or VWF:CB < 10 U dL⁻¹, and/or VWF:RCO < 10 U dL⁻¹, and/or FVIII < 20 U dL⁻¹. Moderate VWD was defined as VWF:Ag 10–30 U dL⁻¹, and/or VWF:CB 10–30 U dL⁻¹, and/or VWF:RCO 10–30 U dL⁻¹, and/or FVIII 20–40 U dL⁻¹ [15]. Children were defined as study participants aged 0–16 years.

Laboratory measurements in VWD

Historic measured VWF and FVIII levels in the Hemophilia Treatment Centers were used as inclusion criteria for the WiN study, and patients with at least one measurement of VWF below 30 U dL⁻¹ or FVIII below 40 U dL⁻¹ (for type 2N), respectively, were included. All participants were asked to give blood for re-evaluation of von Willebrand parameters.

Peripheral venous blood was collected in tubes containing 3.2% (0.109 M) sodium citrate. Subsequently, the tubes were centrifuged twice at $2200 \times g$ for 10 min at room temperature and finally the citrated platelet-poor plasma (PPP) was aliquoted and stored at -80°C .

Plasma levels of VWF:Ag, VWF collagen binding (VWF:CB), VWF activity (VWF:Act) and FVIII were measured centrally (Erasmus University Medical Center, Rotterdam, The Netherlands). Briefly, VWF:Ag level was measured with an in-house ELISA using a polyclonal rabbit anti-human VWF antibody (Dako-Cytomation, Glostrup, Denmark) for capturing and HRP-conjugated anti-human VWF antibody (Dako-Cytomation) for detecting. The VWF:CB level was measured with an in-house ELISA using collagen type 1 for capturing and HRP-conjugated anti-human VWF

antibody for detecting. The VWF:Act assay uses latex particles coated with a monoclonal murine antibody directed against the GpIb α -binding domain of VWF. These latex particles were incubated with the patient plasma (Instrumentation Laboratory B.V, Breda, The Netherlands) and agglutination of the particles, proportionally to the GpIb α -binding activity of VWF, was measured (Sysmex CA-1500, TOA Medical Electronics, Kobe, Japan). The FVIII:C was measured in a one-stage clotting assay (TriniCLOT, Biomerieux, Marcy l'Etoile, France) with FVIII-deficient plasma (Biopool, Umea, Sweden).

Plasma fibrinolytic potential

The fibrinolytic potential of VWD patients was studied using a plasma fibrinolytic potential assay [7,16,17]. Lysis of a tissue factor-induced clot by exogenous tissue-type plasminogen activator (t-PA) was studied by monitoring changes in turbidity during clot formation and subsequent lysis as described previously [7]. Briefly, 70 μ L of citrated PPP was added to 96 wells plate (Falcon; BD Biosciences, Franklin Lakes, NJ, USA) followed by 49 μ L of assay buffer (25 mM HEPES; 137 mM NaCl; 3.5 mM KCl; 1% BSA; pH 7.4). After mixing, 85 μ L of the diluted plasma was added to another 96 wells plate containing 15 μ L assay mixture. The assay mixture included: assay buffer, tissue factor (Innovin, Dade Behring, Marburg, Germany), CaCl_2 , t-PA (Actilyse, Boehringer Ingelheim, Ingelheim am Rhein, Germany), phospholipids (Rossix, Mölndal, Sweden). Plasma samples were measured both in absence and presence of potato carboxypeptidase inhibitor (PCI), a potent inhibitor of activated TAFI [18]. The inhibitory effect of TAFI on the fibrinolytic potential is excluded by adding PCI. The final concentrations in the plasma clot were 1000 \times diluted tissue factor (Innovin), 17 mM CaCl_2 , 30 ng mL $^{-1}$ t-PA, 10 μ M phospholipids and 2 \times diluted

plasma. In the measurements with PCI, the final concentration of PCI in the plasma clot was 30 μ g mL $^{-1}$. Finally, after mixing the diluted plasma with the assay mixture (1400 rpm on a plate shaker for 10 s), 50 μ L paraffin oil (Merck, Darmstadt, Germany) was added to the wells. The plate was then immediately inserted into a 37°C preheated microplate reader (Biotek, Winooski, VT, USA). Optical density was measured at 405 nm every minute for 300 min. The time of the midpoint from the lowest to highest optical density was used as a measure for clotting time, while the time of the midpoint from highest to lowest optical density was used as a measure for lysis time. The clot lysis time (CLT) was calculated as the difference between clotting and lysis time. A representative graph of the fibrinolytic potential assay (optical density plotted against time) is shown in Fig. 1. The intra- and inter-assay variation coefficients were 3.5% and 6.5%, respectively.

In patients with type 3 VWD, low FVIII levels due to an increased FVIII clearance, will theoretically lead to less thrombin generation and subsequently to less TAFI activation and more enhancement of fibrinolysis (shortened CLT). Clot lysis time ratio (CLT ratio) was defined as CLT divided by CLT $^{+PCI}$, thus CLT with TAFI activity divided by CLT without TAFI activity. The CLT ratio was measured to study the effect of TAFI per type of VWD.

Bleeding severity

Bleeding severity was assessed using the Tosetto Bleeding Score (BS) [3,19]. The BS systematically evaluates bleeding symptoms, and accounts for both the number and severity of the bleeding symptoms. The 12 bleeding items are scored on a scale ranging from -1 to 4 points. Higher scores reflect more severe/frequent bleeding. The total for all 12 items results in a BS that can range from -3 (no bleeding) to 45 (severe bleeding).

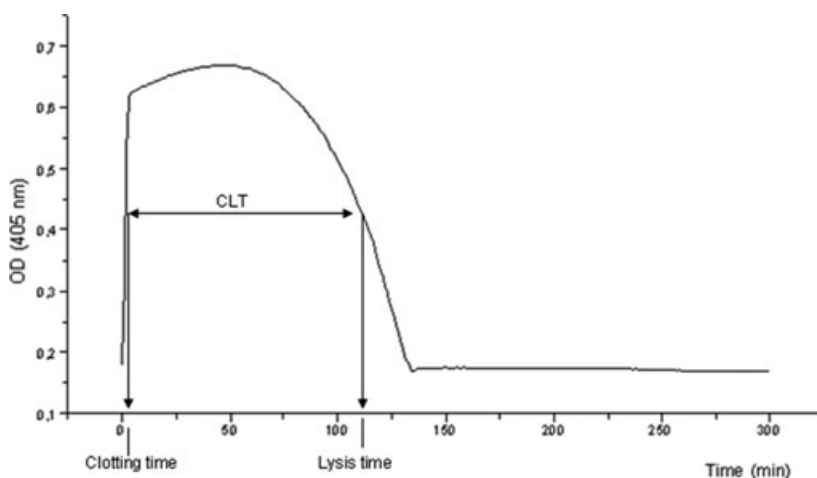


Fig. 1. A representative graph of the output generated by the plasma fibrinolytic potential assay. Clot lysis time (CLT) was calculated as the difference between clotting and lysis time.

For additional analysis, we also used a mucocutaneous bleeding score (BS-mucocutaneous), which accounts for six mucocutaneous bleeding symptoms (epistaxis, bleeding from minor wounds, oral cavity bleeding, gastrointestinal bleeding, bleeding after tooth extraction and menorrhagia).

Statistical analysis

Data were analysed with two objectives. First, determinants of CLT were assessed. The CLT presented a skewed distribution to the right. Due to non-normal distribution, the Mann–Whitney *U* and Kruskal–Wallis tests were used to compare CLT between groups. We used linear regression to model the associations between CLT and its potential determinants. Because the distribution of CLT is skewed, CLT was 10log-transformed. Second, we investigated the association between CLT and BS. Three linear regression models were built with BS or BS-mucocutaneous as dependent variable and 10log-CLT, age, gender, FVIII and VWF:Act as the predictor variables. A *P*-value of ≤ 0.05 was considered statistically significant.

Results

Population WiN study

In the Willebrand in the Netherlands (WiN) study, 806 patients with moderate to severe VWD were included. Plasma was available for 687 VWD patients. Eighteen VWD patients were excluded because of either pregnancy or treatment with desmopressin or FVIII/VWF concentrate 3 days before blood samples were drawn. In 31 VWD patients, no CLT could be determined as the plasma clot did not reach the midpoint from maximum turbidity to clear transition within 300 min. These patients were excluded from subsequent analyses. A flow chart of the study population is shown in Fig. 2.

Table 1 shows the characteristics of the WiN study population. In total, for 638 patients both data on bleeding phenotype and CLT were available; of these 61% were women. The majority of patients were diagnosed as VWD type 1 ($n = 372$, 58%), type 2 was diagnosed in 35% ($n = 222$), type 3 was diagnosed in 4% ($n = 28$) of the included patients, and 3% ($n = 16$) of the patients remained non-classified. VWF:Ag, VWF:CB, VWF:Act and FVIII levels were strongly reduced in our cohort with VWD patients, see Table 1. Median CLT of the study population was 102.5 min [interquartile range (IQR) 92.3–114.0 min], median CLT^{+PCI} was 72.1 min (IQR 65.4–80.9 min).

An association between VWF or FVIII level and BS was found. The median BS was 11 (95% CI 10–11) in ($n = 564$) patients with VWF:Ag levels >10 IU dL⁻¹ and 15 (13–17) in ($n = 74$) patients with VWF:Ag level

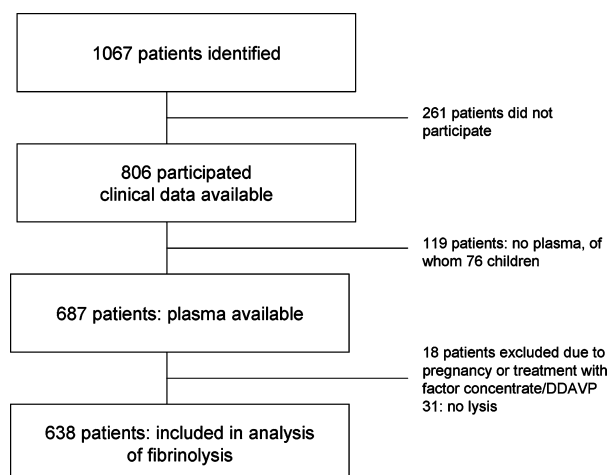


Fig. 2. Flow chart of the WiN study population.

Table 1. Characteristics of the WiN study population.

Total $n = 638$			
Gender	Females ($n, \%$)	389	61%
Age	Median, IQR	45	30–58
VWD type	1 ($n, \%$)	372	58%
	2 ($n, \%$)	222	35%
	2A	92	
	2B	57	
	2M	30	
	2N	15	
	2 not specified	28	
	3 ($n, \%$)	28	4%
	Unspecified ($n, \%$)	16	3%
VWF:Ag	Median U dL ⁻¹ , IQR	29	18–44
VWF:CB	Median U dL ⁻¹ , IQR	22	7–49
VWF:Act	Median U dL ⁻¹ , IQR	22	8–52
FVIII:C	Median U dL ⁻¹ , IQR	50	32–72
Blood group O	$n, \%$	382	60%
VWD severity	Severe VWD ($n, \%$)	253	40%
	Moderate VWD ($n, \%$)	385	60%
Bleeding Score	Median, range	10	–1–35
CLT	Median (min), IQR	102.5	92.3–114.0
CLT ^{+PCI}	Median (min), IQR	72.1	65.4–80.9

IQR, interquartile range; PCI, potato carboxypeptidase inhibitor; VWD, von Willebrand disease; VWF, von Willebrand factor; CLT, clot lysis time.

≤ 10 IU dL⁻¹. Patients with VWF:Act levels >10 IU dL⁻¹ ($n = 473$) or FVIII:C levels >10 IU dL⁻¹ ($n = 605$) had a median BS of 10 (10–11) and 11 (10–12), respectively. A median BS of 14 (13–15) and 18 (15–21) was observed in patients with VWF:Act levels ≤ 10 IU dL⁻¹ ($n = 186$) or FVIII:C levels ≤ 10 IU dL⁻¹ ($n = 33$).

Association between VWF and FVIII levels and CLT

No associations were found between VWF or FVIII level and CLT, see Table 2. Patients in a subgroup with higher FVIII level (>30 IU dL⁻¹) had slightly higher CLTs, although this difference was not statistically significant, *P* for trend 0.097. No associations were found between VWF or FVIII level and CLT^{+PCI}, measuring CLT in the absence of TAFI activity, see Table 2.

	Levels*	CLT (IQR)	<i>P</i> for trend†	CLT+PCI (IQR)	<i>P</i> for trend†
VWF:Ag	0–10	100.3 (90.4–112.5)	0.122	72.5 (65.6–79.1)	0.222
	10–20	99.5 (90.8–109.7)		71.0 (63.3–78.7)	
	20–30	101.5 (91.0–114.2)		71.0 (64.6–79.8)	
	>30	103.8 (94.7–117.6)		73.3 (66.3–82.8)	
VWF:CB	0–10	102.4 (91.3–111.6)	0.316	72.2 (65.0–80.9)	0.822
	10–20	103.9 (93.5–111.2)		72.4 (67.2–79.7)	
	20–30	96.6 (89.4–112.6)		71.0 (64.4–78.5)	
	>30	103.0 (93.6–117.7)		72.1 (65.2–82.2)	
VWF:Act	0–10	100.4 (90.3–110.5)	0.121	71.0 (64.9–78.5)	0.421
	10–20	103.2 (91.8–110.6)		72.5 (66.7–80.0)	
	20–30	103.5 (92.9–115.5)		72.0 (64.5–81.8)	
	>30	103.3 (93.4–117.9)		72.6 (65.9–82.8)	
FVIII:C	0–10	101.9 (90.2–112.2)	0.097	74.6 (67.9–78.9)	0.132
	10–20	102.5 (90.3–111.3)		71.7 (65.4–78.4)	
	20–30	98.5 (90.9–107.4)		68.7 (64.9–78.7)	
	>30	103.4 (92.9–115.0)		72.2 (65.3–82.1)	

*In IU dL⁻¹.

†Clot lysis time and clot lysis time+PCI were 10log-transformed.

Table 2. Association between CLT and VWF/FVIII levels.**Table 3.** Clot lysis time in subgroups of VWD patients.

		N	Median CLT	IQR	* <i>P</i> -value	Median CLT ^{+PCI}	IQR	* <i>P</i> -value
Sex								
Children	Boys	35	90.8	85.3–98.0	0.600	66.4	62.2–74.4	0.771
	Girls	26	90.4	84.8–103.0		68.4	60.4–74.1	
Adults	Males	214	106.2	95.7–118.1	0.008	71.7	65.7–81.5	0.379
	Females	363	101.9	92.8–114.0		72.8	66.6–81.8	
Age	0–16	61	90.6	85.3–100.5	<0.001	67.5	61.0–74.1	<0.001
	16–40	200	99.4	90.9–108.5		70.0	63.6–78.1	
	41–60	244	105.1	93.1–122.4		73.3	65.4–83.6	
	61–86	133	107.0	99.0–117.3		75.4	68.4–83.7	
Type VWD	Type 1	372	101.0	91.5–112.6	0.259	70.4	63.9–78.9	<0.001
	Type 2	221	104.2	94.2–114.5		74.5	68.3–82.0	
	Type 3	28	103.2	89.6–112.0		76.2	69.5–87.4	
Blood group	O	382	101.2	91.9–111.8	0.085	71.5	65.0–80.3	0.100
	Non-O	247	104.0	93.0–115.0		73.4	65.9–81.6	

IQR, interquartile range.

*Mann–Whitney *U* or Kruskal–Wallis test for differences between subgroups.Bold values indicate significant *P*-values.

Determinants of CLT in VWD patients

The CLTs were prolonged in men compared to women with VWD [106.2 (IQR 95.7–118.1) vs. 101.9 (IQR 92.8–114.0) min, *P* = 0.008], see Table 3. In children no gender difference was found [90.8 (IQR 85.3–98.0) vs. 90.4 (IQR 84.8–103.0) min, *P* = 0.600], for boys and girls, respectively, see Fig. 3. The CLT steadily prolonged with age, an increase in age of 10 years was associated with an increase in CLT of 3.3 min (95% CI 2.3–4.4). The CLTs of the children were significantly shorter compared with adult VWD patients [90.6 (IQR 85.3–100.5) vs. 103.7 (IQR 93.2–115.0) min, *P* < 0.001]. No difference in CLT was found between patients with type 1, 2 and 3 VWD. However, a significant difference in CLT+PCI (to exclude the effect of TAFI) between the various types of VWD was observed, see Table 3. Patients with type 3 VWD had prolonged CLTs compared with VWD type 1 and type 2. Within the group of patients with type 2 VWD, patients with subtype 2A had the highest CLT (data not shown).

Association between CLT measured in presence and absence of PCI

Correlation between CLT and CLT+PCI was highly significant (*r* = 0.857, *P* < 0.001), indicating that VWD patients with a high overall CLT also had a high basal, TAFI-independent CLT. To study the contribution of TAFI and TAFI activation on lysis time we measured CLT ratios. In Fig. 4, the CLT ratios per type of VWD are depicted. A significant higher CLT ratio was observed in type 1 compared to type 2 and type 3 VWD patients [median CLT ratio in type 1 VWD 1.45 (IQR 1.37–1.52), type 2 VWD 1.39 (IQR 1.32–1.47), type 3 VWD 1.36 (1.27–1.43), *P* < 0.001].

Effect of fibrinolysis on BS

For both BS and BS-mucocutaneous three models were built to adjust for other factors that could potentially affect the association between BS and

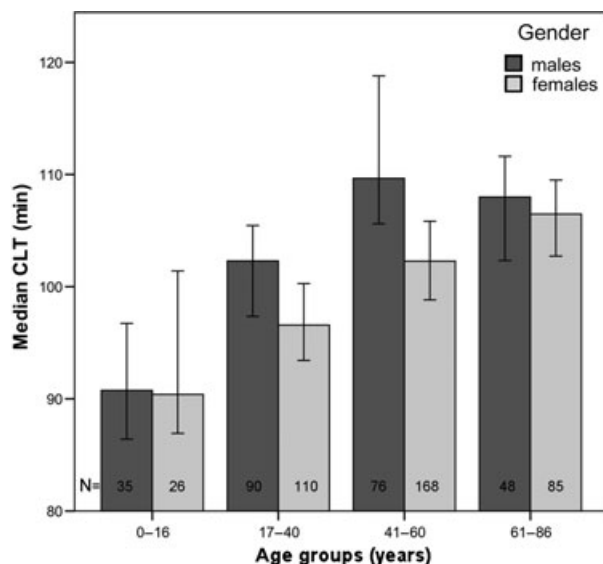


Fig. 3. Clot lysis time per gender and age-group. Children had significantly lower CLTs compared to all other age groups (Mann-Whitney test $P < 0.001$). Only in patients aged 41–60 years a significantly different CLT was found between men and women (Mann-Whitney test $P = 0.006$). Both for men and women CLTs increased significantly with age (Kruskal-Wallis test $P < 0.001$). Error bars: 95% CI.

CLT, including age, gender, VWF:Act and FVIII. The results are summarized in Table 4. An association was found between both BS and BS-mucocutaneous

Table 4. Linear regression models representing the effect of clot lysis time on the Bleeding Score.

<i>n</i> = 638	BS	BS mucocutaneous
	<i>b</i> (95% CI)	<i>b</i> (95% CI)
CLT		
Model I	9.6 (2.8–16.4)	7.9 (3.3–12.4)
Model II	5.8 (–1.2–12.8)	5.4 (0.7–10.1)
Model III	6.5 (–0.3–13.4)	5.3 (0.7–10.0)

Model I: independent variable 10log-CLTs.

Model II: independent variables 10log-CLTs, age and gender.

Model III: independent variables 10log-CLTs, age, gender, VWF:Act and FVIII.

b represents the increase in BS or BS mucocutaneous per min increase of 10log-CLT.

with 10log-transformed CLT [$b = 9.6$ (95% CI 2.8–16.4), and $b = 7.9$ (95% CI 3.3–12.4), respectively]. However, for BS and CLT no significant association was found after adjustment for age, gender, VWF:Act and FVIII ($b = 6.5$, 95%CI –0.3 to 13.4), for BS-mucocutaneous a small regression coefficient was found ($b = 5.3$, 95% CI 0.72–10.0). After additional stratification for type of VWD this association disappeared (data not shown).

Discussion

We did not observe an association between the fibrinolytic potential, measured by a plasma-based clot lysis assay, and bleeding tendency in our cohort of moderate to severe VWD patients. Therefore, our hypothesis that a higher fibrinolytic potential may increase the bleeding tendency in VWD patients could not be confirmed in this study.

In patients with VWD, a large variation in fibrinolytic capacity was observed, which was irrespective of FVIII and VWF level. The fibrinolysis assay we used is an overall measure of the plasma fibrinolytic potential in which the total balance of plasma proteins involved in clot degradation determines the outcome, and a single factor is only of limited importance [7]. The large variability in CLT was also observed earlier in healthy individuals [6,16,17].

We hypothesized that enhanced fibrinolysis might result in a more severe bleeding tendency, leading to an inverse relationship between CLT and bleeding phenotype, assessed by the Tostetto Bleeding Score. We studied mucocutaneous bleeding separately, because of the high fibrinolytic activity in mucosal tissue and the high frequency of mucosal bleeding in patients with fibrinolysis abnormalities [20]. We found a weak, positive association between CLT and BS, however, in adjusted models this association disappeared. This is in line with a previous study in haemophilia patients, in whom an association between clot lysis and bleeding phenotype could not be demonstrated [21].

An explanation for the fact that no association between BS and CLT was found, might be that our

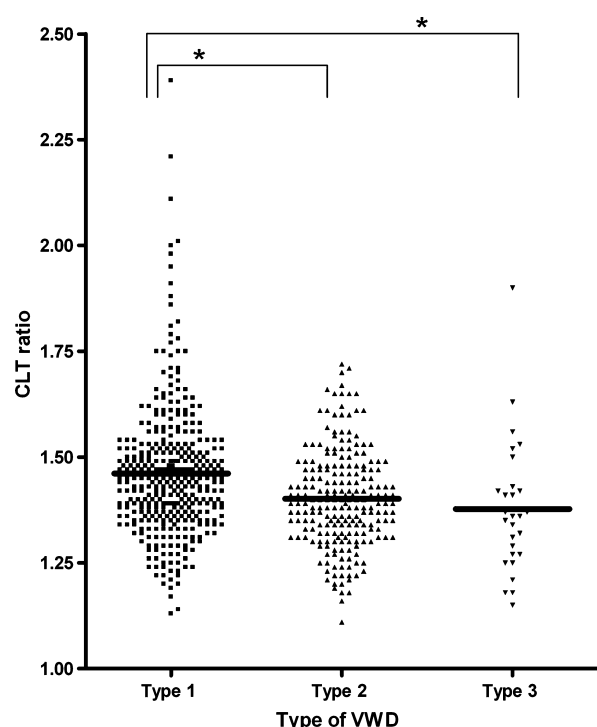


Fig. 4. Clot lysis time ratios in different types of VWD. CLT ratio is defined as CLT (with TAFI activity) divided by CLT^{+PCI} (without TAFI activity). * $P < 0.05$. Bars represent the median CLT:CLT^{+PCI} ratio.

cohort was strictly defined and only patients with moderate or severe VWD i.e. those who have a relatively high BS were included. The selection of the study-group, not including mild VWD patients may have masked the effect of fibrinolysis on bleeding tendency. Mild VWD patients have a larger variability in bleeding phenotype and may be better suited to study the effect of fibrinolysis on bleeding variability. Furthermore, we studied bleeding phenotype by determining the BS in our study [3]. We used the BS because it is a validated method to determine bleeding phenotype in VWD patients. The BS was originally developed by Tosi *et al.* to distinguish between type 1 VWD patients and patients without VWD. This BS has not been used to quantify bleeding symptoms in patients with disorders of fibrinolysis.

The CLT was also measured in presence of PCI, an inhibitor of activated TAFI, to study specifically the role of TAFI. We hypothesized that very low FVIII levels in severe VWD patients might influence the fibrinolytic system because of less TAFI activation, which may in turn lead to differences in bleeding phenotype. The fibrinolytic potential measured in the presence of TAFI inhibitor (CLT^{+PCI}) was indeed increased (shortened CLT^{+PCI}). The anticipated effect of low FVIII activity and concomitant reduced TAFI activation on CLT is not reflected in the clot lysis assay in our study (Table 2). Previous studies showed that FVIII levels were not associated with CLTs in healthy individuals [7]. Furthermore, it was shown that addition of only 0.01 U dL⁻¹ FVIII in severe haemophilia A patients, who are completely lacking FVIII, already resulted in maximal prolongation of CLT, suggesting the effect of FVIII at fibrinolysis is maximal at low concentration [22].

Although we did not find an association between the global plasma fibrinolysis and bleeding tendency, future studies should investigate whether or not the variation in bleeding tendency in VWD might be partly related to individual components of the fibrinolytic system. In the global fibrinolysis assay that we used in this study, exogenous t-PA was added, therefore effects of t-PA or other fibrinolysis protein variations might be masked. Other global fibrinolysis assays or measurement of individual fibrinolysis proteins including α_2 -antiplasmin, PAI-1 or TAFI levels may still be relevant in understanding the variation in bleeding tendency in VWD.

In conclusion, our study showed that the plasma fibrinolytic potential does not influence bleeding tendency in VWD patients and therefore does not explain the variability in bleeding phenotype in VWD.

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Author contributions

FWGL and DR designed the research, analysed and interpreted data, and wrote the manuscript. EMW and KK performed research, analysed and interpreted data, and wrote the manuscript. EPMB, JGB, JCJE, KF, AGB and BLG: designed research, analysed and interpreted data, and critically reviewed the manuscript. MDD: analysed and interpreted data, and critically reviewed the manuscript. All authors gave their consent to the final version of the manuscript.

Disclosures

FWGL is a member of the Dutch haemophilia advisory board of CSL Behring, and attended a round table meeting of Baxter. HCJE received research support from CSL Behring and he has been a teacher on educational activities of Roche. KM and EPMB are members of the Dutch haemophilia advisory board of CSL Behring. J.G. van der Bom has received unrestricted research/educational funding for various projects from the following companies: Bayer Schering Pharma, Baxter, CSL Behring, Novo Nordisk, and Wyeth. In addition, she has been a consultant to Baxter and Wyeth, and she has been a teacher on educational activities of Bayer Schering Pharma. None of the other authors has a conflict of interest to declare.

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